PH.D. THESIS

Serological tests and their applications based on cellular effector functions of immunoglobulins

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Introduction

The human organism is protected by its immune system against external and internal threatening agents. Externally threats can be infectious agents like various microorganisms (viruses, bacteria, monocytes, fungi) and macro-organisms (parasitic worms). An internal danger may be posed by the appearance of autoreactive cells, inappropriate destruction of tumor cells, deposition and inadequate clearance of antibodies.

Myeloid cells express Fc receptors that bind immunoglobulins, thus forming a kind of bridge between the innate and the adaptive immune system, since antibodies are produced by plasma cells belonging to the adaptive system. Fc receptors, upon engagement and cross-linking by immunoglobulins, initiate signal transduction pathways, resulting in phagocytosis, cytokine and chemokine expression, release of inflammatory mediators and of reactive oxygen species.

Antibodies, also called immunoglobulins, have five major classes in the human, IgA, IgD, IgE, IgG, and IgM. Among antibodies, the IgG isotype is found at the highest concentration in human serum and extracellular fluid: 10-20% of the total plasma protein concentration and 70-75% of the total Ig. IgG isotype has four subclasses, IgG1 has the highest concentration in human serum, making up for 60-70% of total immunoglobulin, of which 30-40% is IgG2, 5-8% is IgG3 and 1 to 3% is IgG4. IgG1 is more responsive to soluble protein antigens, IgG2 to bacterial oligosaccharides, IgG3 to protein antigens, and IgG4 to proteins and allergens. IgA is present in the serum as a monomer, is found in the mucosa and in the colostrum as a dimer and constitutes 15% of the total immunoglobulins. There are two subclasses, IgA1 and IgA2. IgA1 is present in the serum at higher concentrations than IgA2. IgA2 is mainly found in the mucosa.

In our work, biological detection of antibodies was performed using myeloid cells, exploiting antibody effector functions mediated by cells. These effector functions were modeled by creating immune complexes to which the cells could bind through their Fc receptors and being adhered to, so that different cellular responses could occur, such as the translocation of NF-κB and the release of reactive oxygen radicals.

Aims of the study

The aim of our research group was to characterize antigen-specific antibodies using cell-based detection methods. A potential problem with immune assays using a secondary antibody is that little or no information is provided on the isotype distribution, glycosylation and the Fc and complement receptor-mediated effects of the detected antibodies. Our solution to this problem was the use of cells instead of secondary antibodies to detect antigen-specific antibodies. This method may also be suitable for the development of a microfluidics-based diagnostic system, since the latter is particularly suitable for the examination of cell interactions.

Our aims were:

- Characterization of cell activation induced by the interactions of Fc receptors and various immobilized immunoglobulins in U937-NFκB cells and neutrophil granulocytes
- Identification of Fc receptors responsible for the activation of U937-NFκB cells and neutrophil granulocytes
- Development of new methods for autoimmune disease diagnostics that exploit cell-mediated immunoglobulin effector functions
- Characterization of cellular adhesion induced by various immobilized immunoglobulins

Applied methods:

- Maintenance of U937 and its transfected variant, U937-NFκB cells
- Purification of leukocytes
- Measurement of cell surface receptors and cell activation by flow cytometry
- Detection of antigen-specific antibodies by protein micro-array techniques
- Measurement of the production of reactive oxygen species produced by leucocytes with fluorescent probe
- Measurement of cell adhesion in a microfluidic system

Results and conclusion I

NF-κB reporter U937 cells were characterized and these cells were used to detect inflammatory immune complexes.

- The U937-NF-κB cell line expresses FcγRI, FcγRII and FcαRI, like the U937 cells.
- We then sought to determine whether cells also produce GFP upon stimulation by hIgG. Cells were incubated with hIgG either bound to the surface or in a soluble form. The immobilized IgG triggered GFP transcription, the surface blocking antibody fragment inhibited this activation.
- U937-NF-κB cells were incubated on a solid surface coated with human immunoglobulin. GFP production reached its maximum at 10 h. GFP production indicated strong activation of cells with IgG3 and IgG4, whereas only a small activation was observed with IgG1. IgG2, IgA, and IgM did not show signs of activation in the cells.
- What FcγR is responsible for cell activation, the GFP production of cells on the surface covered with immobilized anti-FcγRI, FcγRII, FcγRIII monoclonal antibodies. Anti-FcγRI gave a greater response than anti-FcγRII. The cells were incubated with immobilized IgG4 in the case of soluble IgG1, IgG3 or IgG4 in the environment to further enhance cellular activation via FcγRI. SIgG1 and sIgG3 significantly inhibited cell activation while sIgG4 significantly inhibited cell activation only at the highest dose.
- Although IgA alone did not activate the cells, but in combination with IgG3 and IgG4 IgA significantly increased the activation, .
- To test whether cells are able to detect autoantibodies, experiments with RA patients were performed. Serum from four groups was tested where the ACPA-IgG+IgA+ group sera and the complexes generated from serum of the ACPA-IgG+IgA- group activated the cells. Neither ACPAIgG-IgA+ nor ACPA-IgG-IgA- sera activated the cells. Since the reporter cell was suitable for detecting ACPA double positive sera, the U937-NF-κB may be suitable for detecting further inflammatory immune complexes.

Our results indicate that U937-NF-κB cells may be suitable as a diagnostic reporter cell line. The activation of these cells on an immunoglobulin-coated surface was characterized. IgG3 and IgG4 strongly increased the GFP signal, and IgA increased this activity, therefore, the FcγRI and FcαRI receptors are synergistic. We were able to detect ACPA with the reporter cells.

Result and conclusion II

In the inflammatory processes exerted by the Fc receptor, we detected different levels of reactive oxygen species in the neutrophil granulocytes in the cases of the IgG and IgA subclasses, we detected differences also in the induction of the adhesion.

- To compare the FcR expression of the examined neutrophil cells with literature data, they were measured by flow cytometry. The cells expressed the FcγRII and FcγRIIIB receptor, but also slightly the FcγRI.
- To investigate whether FcγRs and FcαRI are capable of producing reactive oxygen species, the solid surface was coated with various immunoglobulin subclasses and neutrophil cells were placed on which DHR-123 was added also. IgG3, IgG4, as well as IgA was a potent stimulator compared to IgG1, which had only a small GFP sign, and IgG2 was a weak stimulator.
- We were curious which FcγR is responsible for cell activation, which was done by DHR-123. Anti-FcγRI and anti-FcγRII were similarly activated and anti-FcγRIII did not activate the cells. Since FcγRI is expressed on resting neutrophils, and cells are activated through it, this finding may complete the previous studies.
- We then examined the cell activation effect of autoimmune complexes with ROS. Immune complexes were antibodies bound to double-stranded DNA from normal and SLE sera . DHR-123-stained cells were able to distinguish between patient and healthy sera.
- The adherence of neutrophil cells to different IgG and IgA subclasses was characterized by optical biosensor. Adherence of cells to IgA was greater than that to IgG. Of the IgA subclasses, IgA1 and of the IgG subclasses, IgG3 gave the highest signal.
- We also examined the adherence of neutrophils to the SLE autoantibodies in a microfluidic device. We used as a negative control, BSA and as a positive control, IgG3. Because we could separate the serum of healthy donors from patients' sera, we have successfully applied our microfluidic system to detect SLE antigen.

In our results, we investigated the activation of primary neutrophil cells with immune complexes. Autoantibodies can efficiently adhere to neutrophil cells, presumably via Fc receptors, and triggered activation can lead to reactive oxygen species and cell adhesion.

Result and conclusion III

Antigen-specific antibodies were assayed for the ability of neutrophil cells potential to bind in a microfluidic device.

- We developed a microfluidic system. First, we have printed HCP2 and VCP2, rheumatoid arthritis-specific citrullinate peptide antigens on a hydrogel-coated Nexterion H slide , as well as human IgG1, IgG2, IgG3 and IgG4 as positive controls, and BSA as a negative control to test the microfluidic device. The PDMSpolymer chamber was placed on the chip, which was generated on the slide.
- In the next step, the U937 cell suspension was placed in the chamber and the cells bound to the immune-complexes. After the incubation was over, methylene blue dye solution was pipetted onto the system, which had a dual role. On the one hand, the non-binding cells were washed out of the system with the dye, and on the other hand, it stained the binding cells. For the evaluation, the printed IgG3 was selected as a control because IgG3 activates the cells most via Fc receptors. In our system, the variable coefficient was below 13% for both VCP2 and HCP2-specific antibodies.
- The serum of a patient diagnosed with rheumatoid arthritis was tested in our system and the reactivity of the sera specific for HCP2 and VCP2 peptides was determined, based on their ability to bind cells to IgG subclasses. More cells bound to VCP2-containing immune-complexes and signals larger were than for HCP2. All serum samples were characterized on the basis of their total pixel intensity for each printed antigen. The antigen-specific IgG levels of these sera were determined by antigen microarray technique and showed a strong positive correlation with the amount of antibody measured and the number of bound cells.

We have introduced a cell-based technique in which a microarray containing the antigen is used together with a microfluidic system carrying capillary channels, through which we could flow the antibodies and cells in the serum at the same time. The capillary force was driving the process without any external impact. It may be advantageous to leave the purification of the cells out of the protocol because the number of receptors in the dextran/ficoll purification technique is altered by purification, as already described.

Summary

Our group has created a biosensor to detect and characterize antigen specific antibodies in the serum. We have genetically modified the U937 cell line with a plasmid encoding NF-κB Responsive Element-driven GFP. We characterized the generated U937-NF-κB cell line‘s expressed Fc receptors and the resulting cellular responses, which were obtained with immobilized antibodies. Human IgG3, IgG4, and IgG1 induced GFP production while IgG2 did not show any activation. IgA alone did not activate the cells, but when used together with IgG3 and IgG4, their effect was significantly increased. FcγR specific monoclonal antibodies were used to determine which Fc receptor was responsible for the cell response. FcγRI had the most prominent effect on the cells, FcγRII had weaker effect, and FcγRIII had no effect. We also confirmed activation through FcγRI with a competitive test. We also performed experiments with real immune complexes with specific peptides and RA sera. The synergistic interaction between IgA and IgG was confirmed. Our results indicate that the U937-NF-κB cell line is suitable for estimating the biological effects of immune complexes.

On the other hand, we also worked with primary neutrophil cells containing reporter cell dyes. Two important steps in the activation of neutrophil granulocytes were investigated: adhesion from Fc activation and the production of reactive oxygen radicals by different immunoglobulin isotypes. Similar to the U937-NF-κB experiments, four IgG subclasses and IgA and its two subclasses were examined. Dihydrorodamine was used to detect the amount of reactive oxygen species formed during cellular activation. The adhesion induced by Fc receptors was measured by an optical biosensor and cellular adhesion was also characterized by a microfluidic device. IgG3, IgG4 and IgA induced the most ROS production of neutrophil cells, and IgG1 had a lesser extent. The effect of IgG2 was negligible. For neutrophil adherence, the IgA subclasses had a more pronounced effect than the IgG isotypes. Adhesion was induced in the following order by the tested antibodies: IgA1, IgA2, IgG3, IgG4, IgG1 and IgG2. Only monoclonal antibodies specific for FcγRI and FcγRII receptors were activatory. Anti-DNA antibody could be detected in serum from lupus patients using neutrophil granulocytes from healthy donors. The microfluidic system we have developed was suitable for the cellular detection of DNA specific antibodies present in SLE sera.

Publication

connected to the thesis

Kecse-Nagy, C* ; Szittner, Z* ; Papp, K ; Hegyi, Z ; Rovero, P ; Migliorini, P ; Lóránd, V ; Homolya, L ; Prechl, J *Characterization of NF-κB reporter U937 cells and their application for the detection of inflammatory immune-complexe* PLOS ONE 11 : 5 Paper: e0156328 , 15 p. (2016) (*: first authors)

Papp, K ; Holczer, E ; Kecse-Nagy, C ; Szittner, Z ; Lóránd, V ; Rovero, P ; Prechl, J ; Fürjes, P *Multiplex determination of antigen specific antibodies with cell binding capability in a self-driven microfluidic system* SENSORS AND ACTUATORS B-CHEMICAL 238 pp. 1092-1097. , 6 p. (2017)

Manusript:

Kecse-Nagy, C ; Sautner, É ; Farkas, E ; Papp, K,; Horváth, R ; Prechl, J *Distinct effects of IgG subclasses on reactive oxygen species generation and cellular spreading of human neutrophil granulocytes underlines complex roles of FcR in regulation of inflammation* (2019)

Conference abstract

Zoltán Szittner, Krisztián Papp, Eszter Holczer, Melinda Herbáth, Csilla Kecse-Nagy, Péter Fürjes, József Prechl *Self-driven microfluidic chambers for a protein microarray cell-binding assay* (2014) Heidelberg EMBL, Microfluidics 2014

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